PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 : C12N 05/02, 11/00, G01N 33/553 — A1

(11) International Publication Number: WO 89/ 06280

(43) International Publication Date: 13 July 1989 (13.07.89)

(21) International Application Number: PCT/US88/02629

(22) International Filing Date: 2 August 1988 (02.08.88)

(31) Priority Application Number: 140,672

(32) Priority Date: 4 January 1988 (04.01.88)

(33) Priority Country: US

(71) Applicant: E.I. DU PONT DE NEMOURS AND COMPANY [US/US]; 1007 Market Street, Wilmington, DE 19898 (US).

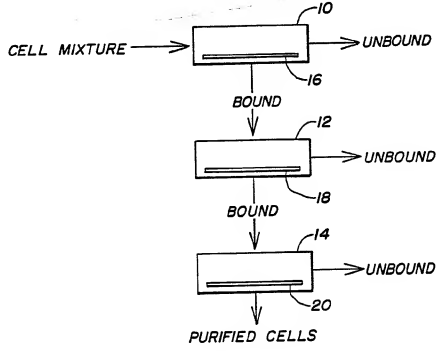
(72) Inventors: PETERSON, Dale, Richard ; 2014 Floral Drive, Wilmington, DE 19810 (US). ARLAUSKAS, Lynn, Ann ; 3400 Pebble Beach Drive, Wilmington, DE 19808 (US).

(74) Agent: WEIGEL, Charles, A., Jr.; E.I. du Pont de Nemours and Company, Legal Department, 1007 Market Street, Wilmington, DE 19898 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), HU, IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, SE (European patent), SU.

Published
With international search report.

(54) Title: MULTIPLE STAGE AFFINITY PROCESS FOR ISOLATION OF SPECIFIC CELLS FROM A CELL MIXTURE



(57) Abstract

Desired cells are positively separated from a mixture of cells using multiple stages (10, 12, 14) of affinity surfaces (16, 18, 20). Bound cells from each surface (16, 18) are removed and subjected to a further surface (18, 20) for further enrichment.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**FR**	France	**ML**	Mali
AU	Australia	**GA**	Gabon	**MR**	Mauritania
BB	Barbados	**GB**	United Kingdom	**MW**	Malawi
BE	Belgium	**HU**	Hungary	**NL**	Netherlands
BG	Bulgaria	**IT**	Italy	**NO**	Norway
BJ	Benin	**JP**	Japan	**RO**	Romania
BR	Brazil	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SN**	Senegal
CH	Switzerland	**LI**	Liechtenstein	**SU**	Soviet Union
CM	Cameroon	**LK**	Sri Lanka	**TD**	Chad
DE	Germany, Federal Republic of	**LU**	Luxembourg	**TG**	Togo
DK	Denmark	**MC**	Monaco	**US**	United States of America
FI	Finland	**MG**	Madagascar		

TITLE

MULTIPLE STAGE AFFINITY PROCESS FOR ISOLATION OF SPECIFIC CELLS FROM A CELL MIXTURE

Field of the Invention

The invention relates to a multistage, positive selection process for separating specific biological cells from a cell mixture. Positive selection is achieved by contacting the cells with an affinity surface having a high affinity for the cell population to be purified.

Background of the Invention

Cell separation techniques have important potential application in cancer therapies, autoimmune disease therapies, and improved diagnostics. For example, cell affinity devices can be used in extracorporeal therapies that may involve the selective isolation, augmentation, and reintroduction to the host of a specific subset population of cells.

Affinity separation of cells refers to process techniques where a particular subset of a population of cells are bound to support surfaces by means of ligands with specific affinity to molecules or structures on the cell membrane. Cells which lack the membrane molecules or structures are not bound to the support surface and can be removed from the population to effect a separation. Cell affinity techniques have been used widely since Wigzell's description of such a process in 1969 [Wigzell and Anderson (1969) J. Exp. Med. 129:23].

Affinity separation processes are commonly used either to deplete cell subpopulations from a mixture or to positively select a specific population from a mixture. The depletion process is much

simpler because the bound cells are simply discarded leaving the desired cells behind. Positive selection is much more difficult both because the desired cells are bound to the support and must be removed without damaging them and because a certain proportion of the undesirable cells bind nonspecifically to the affinity surface and contaminate the desired collected cells.

Affinity cell depletion techniques have found some important applications. Researchers prepare specific cell subpopulations for study by systematically depleting a mixture of various subpopulations of cells. For example, Treleavan et al. (Treleavan et al., 1984, Lancet 1:70-73) have demonstrated that the concentration of neuroblastoma cells in a bone marrow preparation can be reduced by a factor of about 10^6 using multiple depletions with antibody-coated magnetic beads.

Two examples of positive selection techniques are those described by Berenson et al. (J. Immunol. Methods, 1986, 91:179-187) and Gaudernack et al., J. Immunol. Methods, 1986, 91:179-187. Berenson et al. bind biotinylated antibodies to target cells and pass them through a column packed with avidin-coated beads, thereby recovering 64% of a population of human bone marrow cells at a final concentration of 73% when the initial concentration was 7%. Gaudernack et al. use antibody-coated magnetic beads to collect a certain subset of T cells. The initial concentration was 30%, and the positively selected population was 96%; the yield is not mentioned. These purities are not adequate for a large number of attractive applications, such as stem cell transplants, or the preparation of subpopulations for cell biology or immunology studies.

Repeated contacting of cells has been shown to be effective in the depletion of cells from mixtures, but repeated contacting of cells for positive selection of subpopulations has not been reported. This is not surprising since theories of the mechanism of affinity cell binding predict no advantage with multiple stages (Hertz et al., 1985, Biotech. and Bioeng., 27:603-612; Bell et al., 1984, Biophys. J. 45; Grinnell, 1978, Intl. Rev. Cytology, 53:65-144). There remains a need to be able to recover cells with higher yields and higher purities.

Summary of the Invention

Contrary to previous theories of affinity separation of cells (Hertz et al., op. cit.; Bell et al., op. cit.; Grinnell, op. cit.), cell binding to immobilized ligands is reversible. The reversible nature of cell binding to immobilized ligands allows the efficient removal and purification of specific subsets of cells from mixtures using multiple stage affinity processes. These processes surpass in efficiency the performance of previously reported processes.

According to this invention, specific biological cells are separated from a cell mixture by a positive selection process which gives both higher cell purity and higher cell recovery than current processes. The invention uses multiple contacting stages. A cell mixture is contacted with a surface having a high affinity for the desired or target cell population. The affinity surface contains immobilized ligand with high affinity for the target cells. Adherent cells are removed from the affinity surface and then reexposed to a second affinity surface with high affinity for the same target cell in a second stage. The number of stages is increased

until the required cell purity has been achieved. Cell recovery is increased by reexposing the nonadherent cells from each stage to a fresh affinity surface in each additional stage.

Alternatively, the process may be accomplished using counter-current extraction techniques where nonadherent cells at a given stage are reexposed to the affinity surface of the preceding stage.

Brief Description of Drawings

Further advantages and features of this invention will become apparent from the following description wherein:

Figure 1 is a block representation of the multiple stage affinity separation process of this invention for the positive selection of cells;

Figure 2 is a schematic representation of a container with bound ligands which may be used for the stages of Figure 1;

Figure 3 is a schematic representation of magnetic particles coated with ligands that may provide the surfaces for any of the stages of Figure 1;

Figure 4 is a block representation of a counter-current affinity cell fractionation process in accordance with this invention;

Figure 5 is a schematic representation of a multistage counter-current cell separations system; and

Figure 6 is a schematic representation of a multistage batch type cell separation system.

Detailed Description of the Preferred Embodiment

The present invention is an affinity separation process for preparing high purity

fractions of cells from mixtures of cells by repeatedly contacting the cells with surfaces coated with immobilized ligands with specific affinity for the desired subpopulation of cells. Cells, as the term is used herein, may include biological cells of any origin, including prokaryotic and eukaryotic organisms. By way of emphasis, noncellular particles including viruses, mycoplasma, and particles in general are included in this definition and can be purified using the affinity separation process of the invention.

A multiple stage affinity cell separation process or multiple stage positive cell selection process according to this invention is shown in block diagrammatic form in Figure 1. Blocks 10, 12, and 14 represent affinity cell separation devices with affinity contact surfaces 16, 18, and 20, respectively. These devices 10, 12, and 14, as is known in the art, may include bead columns, petri dishes, magnetic beads, fiber arrays, porous membranes, hollow fibers, roller bottles, emulsions, slurries, and the like. The surfaces 16, 18, and 20 in the devices may be formed of any of the materials known to be useful for this purpose and include gels (such as Sepharose), polymers (such as polyarylates, polyesters, polyaldehydes, polyimides, polyvinylpyrrolidones, polyaramides, polyacrylonitriles, polysulfones, cellulosics, ionomers, and fluoropolymers), proteins, lipids, surfactants, glasses, and ceramics. The surface of a device, for example, the bottom of a polystyrene container 22, such as illustrated in Figure 2, is coated with the immobilized ligand 24. The immobilized ligand 24 has affinity for the cell population that is desired to be isolated. The cell

separation devices 10, 12, and 14 may each be the same or differ in type and may number two or more for each stage.

The cell contacting surfaces are coated with a ligand 24 which binds cells with a specific affinity. The immobilized ligand may be, for example, an antibody molecule recognizing a specific antigen on the cell surface. The immobilized ligand could also be a specific ligand molecule, such as a lectin, dye, or a receptor ligand, that is bound by a receptor or ligand-binding molecule on the surface of the cell to be purified. The affinity ligand may also be, for example, biotin, avidin, protein A, an enzyme, an enzyme substrate, or a receptor. Sandwiches or combinations of ligands and ligand-binding molecules may be used. For example, T3-bearing cells may be captured by using a mouse monoclonal antibody specific for T3 and an immobilized antibody specific for mouse immunoglobulin, i.e., the mouse anti-T3 antibody. Molecular spacers or bridges may be used to facilitate interaction of the ligand and ligand-binding molecules. The same or different affinity ligands may be used at each step or stage in the multiple stage separation process.

The affinity ligands may be bound to the cell contacting surface by any of the well-known techniques. For example, physical adsorption, covalent chemistry, physical entrapment, hydrophobic interactions, or Van der Waals interactions may be used. Current techniques for the attachment of proteins to solid supports are reviewed in Affinity Chromatography and Related Techniques, edited by T.C.J. Gribnau, J. Vissen, and R.J.F. Nivand Elsevier, 1982.

The media may be any suitable media that is not harmful to the cells, the ligand, or the ligand-binding molecule. Commonly used media include Hanks balanced salt solutions, RPMI 1640, phosphate buffered saline, Eagle's or Dulbeco's minimum essential media. Other common media and additives are described in. American Type Culture Collection (Rockville, MD) Catalogue of Cell Lines and Hybridomas, 5th edition, 1485, pp. 263-273.

The method of this invention, as depicted in Figure 1, includes placing a mixture of cells, containing desired cells to be separated, in a first medium such that the cells contact the surface 16 and the desired cells become bound to the ligands thereon. Unfortunately some undesired cells also become attached. The cells which do not become attached to the surface ligands are washed from the surface by additional media and discarded. Cells which are bound or adhere to the affinity surface 16 are removed from the affinity surface. The cells may be removed by any well known method including scraping, agitating, fluid shear, use of an elution buffer, or by natural desorption. These removed cells are resuspended in a fresh medium and introduced in the second device 12.

The mixture of removed cells is contacted with the affinity ligand-containing surface 18 in the second device 12. After contact with the affinity surface, the unbound cells are washed out and the bound cells are removed from the surface 18. The removed cells are resuspended in fresh medium and introduced into a third device 14. After contact with the third device's affinity surface 20, the unbound cells are again washed out and the bound cells are removed to constitute the purified cells.

This process of positive selection and elution of the positively selected cells can be repeated as many times as necessary to achieve the desired degree of purity and yield.

In its simplest form, the method of the invention may use petri dishes coated with an appropriate ligand (antibody) recognizing a molecular marker specific for the cell population to be purified. Cell mixtures are allowed to settle to the bottom and bind to the surface of the antibody-coated dish. Nonadherent cells are then washed away. The adherent cells are scraped and the free scraped cells are resuspended in fresh media. The resuspended cells are then poured into a fresh antibody-coated dish, and the procedure repeated until the adherent cells are of the desired purity.

Alternatively, the method of this invention may immobilize ligands 28 on magnetic particles 30, as seen in Figure 3, in a known manner. The particles are placed in a container 32 in a fluid medium 36 suitable for the ligands and cells to be separated. Magnets 34 are moved into position along the sides of the container after the cells to be separated are mixed with the magnetic particles 30 for binding to the ligands 28 thereon. This secures the particles 30 with the attached desired cells to the side walls of the container 32 so that the unattached cells and medium may be discarded and the secured particles washed thoroughly. The container is again filled with a fresh medium and the magnets withdrawn (or deenergized) to release the particles. The desired cells are allowed to be released from the particles by natural desorption. The released cells are collected and introduced into a second container (not shown except as block 10, Figure 1) with a fresh

medium and ligand-coated magnetic particles. The procedure described above is repeated to further increase the purity of the cells.

The yield or recovery of the desired purified cell population can be increased by using a counter-current processing technique in which multiple stage positive selection is combined with multiple stage depletion. Figure 4 is a schematic representation of a counter-current multiple stage positive selection method for the purification of cells. Blocks 40, 42, 44, 46, and 48 are affinity cell separation devices with affinity contact surfaces 50, both devices and surfaces being of the types described above. The cell separation devices for batch types may each be the same or differ in type and there may be two or more total devices in number. In Figure 4, five devices are illustrated.

The affinity surfaces are coated with a ligand, of the type described above, which binds the cell population to be purified with a specific affinity. In a batch type operation, the operation of the several devices 40 to 48, inclusive, is similar to that previously described. The positively selected cells removed from the affinity surfaces 50 are each moved down the chain, as represented by the lines 52, to a different contacting surface 50 in one of the devices 40 to 48, inclusive. In each case, the selected or desired cells are placed into a fresh media for contact with the new surface 50. After each use, the particular device 40 to 48, inclusive, may be removed and replaced by a fresh device or more preferably a fresh affinity surface is positioned within the device.

Conversely, the unbound cells that are removed with the media from the respective devices 40

to 48, inclusive, travel upwardly in the schematic of Figure 4, as represented by the lines 54. A wash solution 56 is introduced into the lower device 48. Purified cells are removed from device 48. The depleted cell mixture is removed from the upper device 40 through the line 58. Cells to be separated are introduced into the middle device 44 as represented by line 60.

Thus in an illustrative application of the batch method, cells to be separated are passed in a mixture into the device 44. After contact with the affinity surface 50 within the device 44, the unbound cells are removed by the media from the wash line 54 and introduced into device 42 for contact with its affinity surface 50. The bound cells are removed from the affinity surface 50 within device 44, resuspended in media and applied to device 46 for contact with its affinity surface 50. Next the unbound cells from device 42 are applied via stream 54 to device 40 for contact with its affinity surface 50 and the bound cells are resuspended and applied to device 44 for contact with its affinity surface 50. The unbound cells from device 40 pass through the outlet and constitute the depleted cell mixture, while the cells bound in device 40 are resuspended in media and applied to device 42 for contact with its affinity surface 50. Depending on the yield required, the bound cells in device 42 are resuspended and applied to device 44, thence to device 46 and so on.

In the meantime, cells not bound to the surface 50 in device 46 are removed by wash line 54 and introduced into the device 44 for contact with its affinity surface 50. Conversely, bound cells are removed from the affinity surface 50 of device 46,

resuspended in media and applied to device 48 for contact with its affinity surface 50. The unbound cells in device 48 are applied via stream 54 to device 46 for contact with its affinity surface 50. The bound cells in device 48 are removed from the affinity surface 50 in device 48 and passed to the outlet 50 as highly purified cells and with a high yield as described. The several batch cycles may be continued to further increase cell yield as desired.

The counter-current batch operation requires repetitive operations at each of the stages. Following each application and removal of cells, a stage must be cleaned and resupplied with an appropriate contacting surface. The batch operation procedure described above provides highly pure desired cells as well as a high yield.

The same kind of counter-current method may be used in a continuous flow device such as a vertically oriented column 90 of suitable material such as glass as seen in Fig. 5, with media entering near the bottom at an inlet 92 and leaving near the top through an outlet 94. Cell mixtures are introduced into the column through an inlet 102 between the media entrance and exit ports. Appropriate surfaces, coated with affinity ligand, such as provided by particles denser than the media or cells, are introduced at the top of the column as at 96 and allowed to settle slowly down through the column 90 to contact the cells rising with the media. Cells which bind to the particles will be pulled down through the column until they desorb from the particles. Nonspecifically bound cells desorb faster than cells bound specifically by affinity ligand. Cells which are bound to particles long

enough will be carried out of the bottom of the column through a valve 98 and exit outlet 100. If the particles settle slowly enough to remain in the column while specifically bound cells to bind, desorb, and bind again more than once (an hour or more) then this device will be an effective multistage counter-current cell separation device. The media and particles must be selected accordingly. If bouyant particles are used, the media flow direction must be switched and the particles introduced at the bottom of the column.

In still another alternative, a batch type process such as depicted in Fig. 6 may be employed. In this schematic, a plurality of affinity surfaces, which may be any of the types described hereinbefore are depicted by the reference numerals 80, 82, 84, 86 and 88. Each affinity surface represents a stage which would be some type of a receptacle or container for holding the media associated with that particular stage. The surfaces are grouped in stages or clusters of two and three surfaces to accommodate appropriate interchange therebetween. Cell mixtures are introduced, as denoted by the arrow at 81, to the first stage 80. After contact with the affinity surface 80 of the first stage, the unbound cells, depicted by the numeral 1N, are transferred and introduced into the upper surface 82 of the second stage. The bound cells are removed from the affinity surface 80 and transferred, as depicted by the line 1A, to the lower affinity surface 82 of the second stage. Unbound cells from the upper stage 82 are passed to the third stage upper affinity surface 84. Bound cells are removed from the upper surface 82 and passed, as depicted by the line 2A, to the middle affinity surface 84. Likewise unbound cells from the

lower affinity surface 82 are passed, as denoted by the line 3N, to the mid-affinity surface 84. Finally bound cells are removed from the lower surface 82 and passed onto the lower affinity surface 84 as noted by the arrow 3A.

Unbound cells from the upper third stage affinity surface 84 are passed, as denoted by the line 4N, to waste. Bound cells from the same stage are removed, as denoted by the line 4A, and introduced to the upper affinity surface 86 of the fourth stage. Similarly the unbound cells from the middle third stage surface 84 are passed, as denoted by the line 5N, to the upper fourth stage affinity surface 86. Bound cells are removed from the middle affinity surface 84 and passed, as denoted by the line 5A, to the lower fourth stage affinity surface 86. To complete the cycle, unbound cells from the lower third stage affinity surface 84 are passed to the lower fourth stage affinity surface 86 via the line 6N whereas bound cells on the lower third stage affinity surface 84 are passed as denoted by the line 6A to a collection device where the high purity desired cells are collected. This sequence repeats itself to achieve the desired yields. Thus unbound cells from surfaces 86 are passed via lines 7N or 8N to appropriate surfaces 88 of a further stage as shown. Bound cells are passed via lines 7A or 8A to surfaces 88 of this further stage as shown. The fifth stage lower surface 88 provides high purity cells as denoted by line 11A; conversely, the line 9N passes to waste media depleted of the desired cells.

Example 1

Attachment of Antibodies to a Culture Dish Surface

The following procedure was used to bind antibodies to the surface of a culture dish. Goat

anti-rat IgM antibody was immobilized on polystyrene tissue culture dishes at a concentration of 0.1 $\mu g/cm^2$ using the following procedure.

A volume of 0.3 ml of a carbodiimide solution (0.05 g of carbodiimide hydrochloride per ml of 0.1 M sodium acetate pH 4.8) and 0.3 ml containing 0.96 µg goat anti-rat IgM per ml of 0.1 M sodium acetate pH 4.8 were added to each 35 mm culture dish well. The dish was incubated with rocking for 60 minutes at 25°C. The wells were washed 3 times with 3 ml of PBS. A second or capture antibody was added in 3 ml of PBS at a concentration of 3 µg/ml to each dish. The dish was again incubated at room temperature for 1 hour without mixing. Excess antibody was rinsed off with two 3 ml aliquots of PBS and one aliquot of PBS containing 1% heat inactivated fetal calf serum (FCS) (Gibco). The remaining protein binding sites on the plate were blocked or quenched by adding 0.1% bovine serum albumin (BSA) in 3 ml of PBS to the dish. The dish was then incubated for 30 minutes at room temperature without mixing. The dish was finally rinsed 3 times with aliquots of 3 ml PBS. In one case the second antibody was that designated 187.1, was specific to mouse immunoglobulin, and was obtained from John McKearn (E. I. du Pont de Nemours and Company, Glenolden, PA). In another case it was the mouse immunoglobulin designated 7D4.

Example 2

Multiple Stage Planning

An example of affinity cell selection by multiple stage panning is detailed below. Mouse CTLL cells (American Type Culture Collection #P1B-214), which are in this case the target cell to be purified, were mixed with human HUT-102 cells

(American Type Culture Collection #P1B-162) at a resultant concentration of 10% target CTLL cells. Polystyrene tissue culture dishes were coated with an IgM monoclonal antibody (mAb) specific for the IL2 receptor present on the surface of the CTLL cells. The mAb is designated 7D4 and was obtained from Tom Waldmann, NCI, Bethesda, MD. The antibody was attached to the polystyrene surface using the procedure given in Example 1.

The CTLL and HUT cells were collected by centrifugation in at 15 ml centrifuge tube for 10 minutes at 1000xg. The media was decanted and the cells were then resuspended in Iscove's medium (Gibco) containing 15% fetal bovine serum (FBS). The suspended cells were then poured into the mAb 7D4-containing petri dish, and incubated at 22°C for one hour. The nonadherent cells were removed by rinsing the petri dish surface with PBS. The adherent cells were scraped off the petri dish surface with a PVC scraper and resuspended in Iscove's medium containing 15% FBS. The resuspended adherent cells were then poured into a fresh petri dish similarly coated with the same anti-IL2 receptor mAb (7D4). Following washing to remove the nonadherent cells, the adherent cells were again removed by scraping. The scraped cells were resuspended and captured in a third anti-IL2 receptor mAb 7D4-containing petri dish. Again the nonadherent cells were removed by rinsing.

The adherent cells were removed by scraping, resuspended and analyzed both by flow cytometry and by limiting dilution assay. Flow cytometry showed that greater than 99% of the adherent cells were CTLL cells while the limiting dilution assay showed a ratio of HUT to CTLL cells of 1:200 (99.5% CTLL

cells). The limiting dilution assay was carried out by diluting the adherent cells to various numbers per well in a 96-well plate containing IL2-free media. Under such conditions the contaminating HUT cells are able to grow, whereas the CTLL target cells, which require IL2 for growth, are unable to grow (Gillis and Smith, 1977, Nature 268:154-156). Growth was seen in half the wells initially containing 100 cells. Thus, the limiting dilution assay showed approximately 0.5 HUT cells per 100 total cells (HUT plus CTLL). Thus, the three-stage affinity cell selection or panning procedure resulted in the target CTLL cells being concentrated from 10% to 99.5%. The yield from this three-stage panning procedure was 14%.

Example 3

Affinity Selection of Surface Immunoglobulin-bearing Spleen Cells from Mouse Spleen Homogenates

Cells were obtained from the spleen from BALB/c mice. The cells were suspended by scrubbing the spleen against a stainless steel screen and rinsing the screen with PBS. Clumps of cells were removed by filtering the suspension through cotton. The cells were washed and resuspended in cold Iscove's medium with 15% FCS at a concentration of 10^6 cells/ml.

In the first stage of the separation, the cells were added to dishes containing immobilized antibody as described above in Example 1 at 2 x 10^5 cells/cm^2 and incubated at 4°C for 1 hour. The nonadherent cells were rinsed away using 8 aliquots of 3 ml PBS containing 1% FCS. The adherent cells were scraped and resuspended in cold Iscove's medium containing 15% FCS. In the second stage of the separation the removed cells were added to fresh

dishes at 3 x 10^5 cells/cm^2 for 1 hour at 4°C. The nonadherent cells were rinsed away as above and the adherent cells were scraped and resuspended in Iscove's containing 15% FCS.

The purity of the resultant cells were assayed by staining the purified cells with fluorescein-labeled 187.1 mAb and measuring the fluorescence using an Ortho Spectrum flow cytometer. The starting mixture was 39% 187.1 positive while the purified cells were over 99% 187.1 positive.

Example 4

Purification of CTLL Cells From Contaminating Bacteria

In an unusual application, bacteria-free cells were isolated from a cell culture contaminated with bacteria. In one experiment, 5 x 10^8ml Bacillus pumilus were added to a 10^6/ml CTLL culture at room temperature. The CTLL cells were isolated using three stages of positive cell selection on culture plates coated with mAb 7D4. The mAb 7D4 was attached to the culture plate as described in Example 1. The three-stage positive selection procedure was performed as described in Example 2. The adherent cells obtained following the three-stage positive selection procedure were analyzed for the presence of contaminating bacteria using a limiting dilution assay. The adherent cells were diluted into wells and cultured. The affinity-purified CTLL cells diluted up to 100 per well grew normally and were bacteria free. The initial concentration of CTLL cells of 0.2% were thereby increased to at least 98.8% by the process. This is a new method for preparing bacteria-free cultures of mammalian cells.

Example 5

Multiple Stage Particle Technique

The magnetic particles used by Gaudernack et al. (op. cit.) are useful for affinity cell separations. By using multiple stages, however, their performance was greatly improved.

Magnetic polystyrene particles of 4.5 micron diameter (Dynal M-450) were coated with mAb 7D4, which is specific for the mouse IL2 receptor, using the following procedure 10^7 magnetic particles and 1.0 $\mu g/cm^2$ mAb 7D4 were added to 1 ml PBS, where the area (cm^2) is the calculated surface area of the magnetic beads assuming they are solid spheres. The mixture was incubated for 2 hours 25°C and mixed frequently. The magnetic particles were washed 2 times with aliquots of 1 ml PBS by retaining the particles with a magnet. 2 mg/cm^2 of BSA in 1 ml PBS was added to the magnetic particles. The sample was mixed to resuspend particles and then incubated 30 minutes 25°C. The magnetic particles were washed 2 times with aliquots of 1 ml PBS by retaining the particles with a magnet. The particles were suspended in Iscove's media containing 15% FBS at a concentration of 10^7 particles/ml.

A mixture of 10% CTLL cells (the target cell to be purified) and 90% HUT-102 cells, at a concentration of 10^6/ml and 10^7/ml, respectively, was added to the 7D4-coated magnetic particles and incubated for 20 minutes at room temperature. The nonadherent cells were washed away by retaining the particles and attached cells with a magnet.

The adherent cells were removed from the particles by natural desorption over a two hour period at room temperature. The desorbed cells were

collected and resuspended with fresh mAb-coated particles for 20 minutes at room temperature.

The purified adherent cells were analyzed by limiting dilution and flow cytometry. Flow cytometry showed greater than 99% CTLL cells while the limiting dilution assay showed greater than 99.97% purity of CTLL cells.

Example 6

Counter-Current Process

The multistage process described above in Example 5 gives excellent purity at the expense of yield. By combining multistage positive selection with multistage depletion, high purity and high yield can both be achieved. 10% CTLL cells were mixed with 90% HUT-102 cells, at a resultant concentration of 10^6/ml and 10^7/ml, respectively, in Iscove's medium containing 15% FBS. The mixture was poured into a petri dish, with appropriate mAb capture reagent on the dish bottom, as described in Example 1, and incubated for 1 hour at room temperature. The nonadherent cells were resuspended in media and poured into a fresh mAb-coated dish. The adherent cells from both dishes were scraped, resuspended, and then poured into a third mAb-coated dish. The final adherent cells were removed and analyzed by flow cytometry and limiting dilution assay. The first dish adherents were 58% CTLL cells with a yield of 50%; the nonadherents of the first dish were 2% CTLL with a yield of approximately 50%. The adherents from the second dish were 15% CTLL cells with a yield of 60%. The adherents from the third dish were 80% CTLL cells with an overall yield of 40%. A corresponding two-stage process without the counter-current treatment gave 86% purity of CTLL cells with 25% yield.

What is claimed is:

1. An affinity method of providing high purity fractions of desired cells by separating the desired cells from a mixture of cells in a first media using plural surfaces having ligands, with a high affinity for the desired cells, immobilized thereon comprising the steps of:

(a) contacting one of the surfaces with the first media mixture of cells to permit some of the desired cells to be bound to the one surface,

(b) separating the unbound cells and the first media from the bound cells,

(c) removing the bound cells from the one surface, and resuspending them in a second media,

(d) contacting a different one of the surfaces with the second media to permit some of the removed cells to be bound thereto,

(e) separating the unbound cells and the second media from the different surface, and

(f) removing the bound cells of step (d) from the different surface to provide the high purity fraction of desired cells.

2. The method as set forth in claim 1 which includes the additional steps of resuspending the removed cells of step (f) in a third media and repeating steps (d) and (e) with a third one of the surfaces with the removed cells of step (f).

3. The method as set forth in claim 2 wherein the surfaces each comprise the bottom of a different container.

4. The method as set forth in claim 1 wherein the surfaces each comprise the bottom of a different container.

5. The method as set forth in claim 2 wherein the surfaces comprise the surfaces of magnetic

particles and the unbound cells and media are separated from the bound cells by separating the magnetic particles therefrom, desorbing the bound cells from the magnetic particles, and resuspending the desorbed cells together with fresh ligand immobilized magnetic particles in a different media.

6. The method as set forth in claim 1 wherein the surfaces comprise the surfaces of magnetic particles and the unbound cells and media are separated from the bound cells by separating the magnetic particles therefrom, desorbing the bound cells from the magnetic particles, and resuspending the desorbed cells together with fresh ligand immobilized magnetic particles in a different media.

7. The method as set forth in claim 1 wherein both unbound and bound cells from each contacting step are separately resuspended and contacted with additional affinity coated surfaces in a counter-current manner until the number of unwanted cells in the bound fraction and the number of desired cells in the unbound fraction have reached predetermined low values.

8. The method as set forth in claim 2 wherein both unbound and bound cells from each contacting step are separately resuspended and contacted with additional affinity coated surfaces in a counter-current manner until the number of unwanted cells in the bound fraction and the number of desired cells in the unbound fraction have reached predetermined low values.

9. The method as set forth in claim 2 wherein the surfaces comprise the interior wall of roller bottles.

10. The method as set forth in claim 1 wherein the surfaces comprise the interior wall of roller bottles.

11. The method as set fort in claim 1 wherein each of the surfaces has a different ligand, each ligand having a different affinity for the desired cells immobilized thereon.

12. An affinity method of providing high purity fractions of desired cells by separating the desired cells from a mixture of cells in a first media using plural surfaces having ligands, with a high affinity for the desired cells, immobilized thereon comprising the steps of:

flowing media in a first sense,

introducing particles to provide the surfaces into the media, the particles having a density relative to that of the media to move in a second sense opposite the first sense,

introducing the cell mixture into a mid-portion of the flowing media, and withdrawing the undesired cells and media at the downstream end of media flow,

withdrawing the particles and desired cells bound thereto at the upstream end of media flow.

13. The method of claim 12 wherein the particles are more dense than the media and the flow is upward.

14. The method of claim 12 wherein the particles are less dense than the media and the flow is downward.

15. An affinity method of providing high purity fractions of desired cells by separating the desired cells from a mixture of cells in a first media using plural surfaces having ligands, with a high affinity for the desired cells, immobilized thereon comprising the steps of:

(a) contacting one of the surfaces with the first media mixture of cells to permit some of the desired cells to be bound to the one surface,

(b) contacting a second surface with the unbound cells from the one surface,

(c) contacting a third surface with the bound cells from the one surface,

(e) contacting a fifth surface with the bound cells from the second surface and the unbound cells from the third surface,

(f) contacting a sixth surface with the bound cells from the third surface,

(h) contacting a seventh surface with the bound cells from the fifth surface and the unbound cells from the sixth surface,

(i) contacting an eighth surface with the bound cells from the seventh surface,

(j) and removing the bound cells from the sixth and eighth surfaces to provide desired cells of high purity and yield.

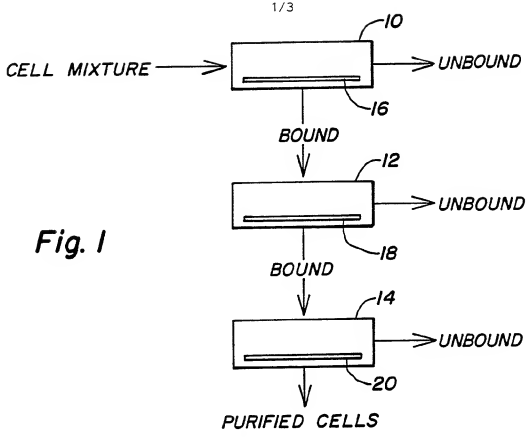


Fig. 1

Fig. 2

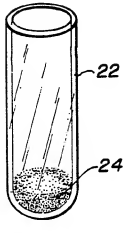


Fig. 3

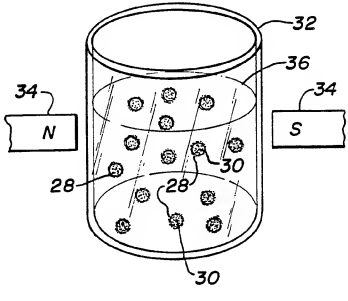


Fig. 4

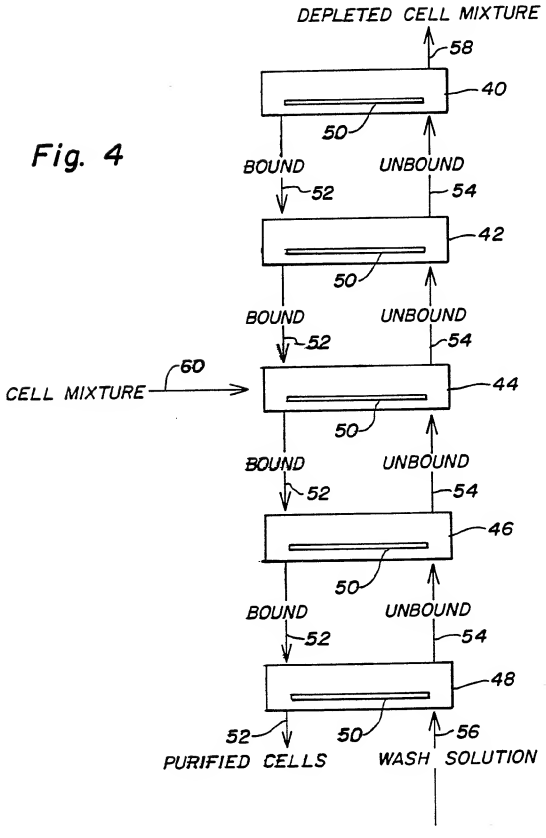

DEPLETED CELL MIXTURE
58
40
50
BOUND
52
UNBOUND
54
42
50
BOUND
52
UNBOUND
54
60
CELL MIXTURE
44
50
BOUND
52
UNBOUND
54
46
50
BOUND
52
UNBOUND
54
48
52
50
56
PURIFIED CELLS
WASH SOLUTION

Fig. 5

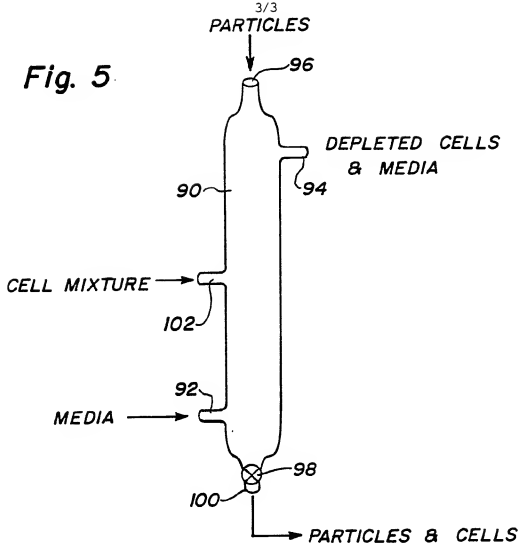
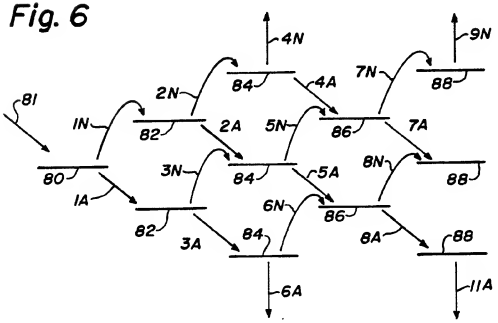

PARTICLES
96
DEPLETED CELLS
& MEDIA
90
94
CELL MIXTURE
102
92
MEDIA
98
100
PARTICLES & CELLS

Fig. 6


4N
9N
81
2N
84
4A
7N
88
1N
82
2A
5N
86
7A
80
3N
84
5A
8N
88
1A
6N
86
82
3A
84
8A
88
6A
11A

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/02629

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C12N 05/02; C12N 11/00; G01N 33/553

U.S. CL. 435(240.243); 530(811); 436(526)

II. FIELDS SEARCHED

Minimum Documentation Searched 7	
Classification System	Classification Symbols
U.S.	436/824, 526; 530/811 435/240.243

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

Computer searched CAS 1967-1988, Biosis 1969-1988, APS 1975-1988.

See Attachment on Search Terms

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
Y	Schleicher, J. "Multisurface stacked plate Propagators" in Kruse, P. et al Tissue Culture, Academic Press, NY, USA 1973, pages 333-338.	1-15
Y	US, A, 4710472 (Saur et al)01 December 1987. See Whole Document.	1-15
Y	JOURNAL OF IMMUNOLOGICAL METHODS 90, 1986. North Holland, Amsterdam. Gaudernack et al "Isolation of Pure functionally active CD8+ T cells" pages 179-187.	1-15

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
15 December 1988	28 FEB 1989
International Searching Authority	Signature of Authorized Officer Lori Y. Beardell
ISA/US	Lori Y. Beardell

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	JOURNAL OF IMMUNOLOGICAL METHODS 91, 1986. Berenson et al. "Positive selection of viable cell populations using avidinbiotin immunoadsorption". pages 11-19.	1-15
Y	US, A, 4452773 (Molday) 05 June 1984. See Whole Document.	1-15
Y	US, A, 3947352 (Cuatrecasas et al) 30 March 1976. See Whole Document.	1-15

Attachment to PCT/ISA/210
Part II. FIELD SEARCHED

Search terms

affinity separation
positive selection
cell affinity, separation or purification
magnet
multiple or serial separation